EVALUATION OF ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECT OF PUNICALAGIN (POMEGRANATE EXTRACT) WITH SCALING AND ROOT PLANING (SRP) AND SRP ALONE ON IL-1 BETA AND SUPEROXIDE DISMUTASE LEVELS IN PATIENTS WITH CHRONIC PERIODONTITIS

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH II

PERIODONTICS

MAY 2018

CERTIFICATE

This is to certify that **Dr. TAMIL SELVAN K,** Post Graduate student in the Department of Periodontics, J.K.K. Nattraja Dental College and Hospital, Komarapalyam has done this dissertation titled **"Evaluation of anti-inflammatory and antioxidant effect of punicalagin (pomegranate extract) with Scaling and Root Planing (SRP) and SRP alone on IL-1 beta and superoxide dismutase levels in patients with chronic periodontitis"** under my direct guidance during his post graduate study period 2015-2018.

This dissertation is submitted to **THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY** in partial fulfillment of the degree of **MASTER OF DENTAL SURGERY, BRANCH II – Periodontics.**

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ACKNOWLEDGEMENT

I am greatly indebted to ***Dr. S. Thangakumaran****, my Professor and Head of the Department, Department of Periodontics, J.K.K. Nattraja Dental College and Hospital, Komarapalayam, for his strenuous and dedicated efforts towards the post graduate students and for his invaluable guidance, support and encouragement throughout my post graduate study.*

I would like to extend my heartfelt gratitude to Professor ***Dr. A. Sivakumar****, Principal, J.K.K. Nattraja Dental College and Hospital, for his kindness in allowing me to utilize the facilities in the college.*

I am also highly obliged to my former head of the department ***Dr. Sugumari Elavarasu*** *for her valuable suggestions, enthusiastic support and constant encouragement throughout my study. Thank you very much mam for guiding me in my thesis work and for educating and motivating me. I am indebted for you forever for all the considerations you have shown towards me, mam.*

I sincerely thank ***Dr. T. Arthiie*** *Reader, who inspired me in every phase of my professional life. Her profound knowledge, patience and perseverance and her incessant encouragement, guidance and support had benefited me and my colleagues in every facet of our academic life.*

I also thank ***Dr. P K Sasikumar*** *Reader, for his guidance and support throughout my entire academic period.*

I also thank ***Dr. M. Thuloth Mangai*** *tutor, for her help and continuous encouragement throughout my entire academic period.*

I am extremely thankful to ***Mr. K. Karthikeyan ARM Lab, Erode*** *for guiding me in working with Biochemical Analysis.*

My heartful thanks to my dear seniors and colleagues, ***Dr. Vijaya kumar P, Dr. Lakshmi Mohandas, Dr. Shiva shangkharii, Dr. Dhivya R, Dr. Fairlin P, Dr. Sree Lakshmi P, Dr. Sugirtha C*** *and all the non-teaching staff's and my department sisters* ***Mrs. Malathi, Mrs. M. Maruthaveni, Mrs. R. Hemalatha and Mrs. R. Vashanthi*** *for their kind help during my postgraduate period.*

I would like to thank all of my patients for their kind cooperation.

Words are not enough to express my sincere gratitude to my dear father ***Mr.Kumar P,*** *mother* ***Mrs.Gangadevi k*** *and my lovable sister* ***Ms.Gayathiri K*** *without whom I would not have been able to reach this height. Above all, it is my privilege to thank my everloving friends* ***Mr. Gowtham M, Mr. Anto Jeffry I, Mr. Naveen S, Mr. Prem SB and Mr. Vignesh Kumar VM*** *for their sacrifice, support and constant encouragement.*

Finally, without the grace of the ALMIGHTY this possibility would have been just impossible.

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Periodontitis is an inflammatory condition which results in the destruction of the tooth supporting structures. The mechanisms underlying this destructive process involves both direct tissue damage resulting from plaque bacterial products, and indirect damage through bacterial induction of the host inflammatory and immune responses. This host response involves complex interactions between cells, the extracellular matrix and circulating cytokines.[1]

The elimination of periodontopathogens containing biofilm remains the first goal of periodontal therapy. Although mechanical debridement such as Scaling and Root Planing (SRP) reduces the level of subgingival bacteria and thereby inflammatory mediators, it does not completely eliminate the pathogens that resides deep into the connective tissue.[2]

The limitations of conventional periodontal therapy led to the emergence of many chemical agents such as antiseptics, NSAIDS and antibiotics. Drugs were used both systemically and locally. Systemic delivery requires the administration of larger doses to obtain required concentration of drug at the site of disease. This has led to the development of bacterial resistance, drug interactions and inconsistent patient compliance.[3] In order to overcome the drawbacks of systemic administration, local delivery system was introduced.

In the Local Drug Delivery systems, the drug can be delivered to the site of disease activity at a required concentration and can facilitate prolonged drug delivery. Although different chemical agents are available in different forms such as gels, strips, microcapsules, fibres and films, search for alternative products continues and natural phytochemicals isolated from plants are considered as good alternatives to synthetic chemicals.[4]

Among the many herbs available, Pomegranate extracts have been reported to have many beneficial health effects, exhibiting antioxidant, anti-inflammation, antiproliferative, and DNA repair activities, which are generally attributed to the high polyphenol content.[5]

Cytokines are soluble proteins produced by the structural and inflammatory cells, into extracellular fluid, where they exert their effects on the same cells or on neighbouring cells by interacting with specific receptors. The role of cytokines in maintenance of tissue homeostasis requires delicate balance between catabolic and anabolic activities.

The inflammatory mediators and cytokines manifest potent pro-inflammatory and catabolic activity and may play a key role in local amplification of the immune response as well as periodontal tissue breakdown. Among the chemical mediators released by the host cells in response to inflammatory stimulus, IL-1 beta is thought to play a major role in the pathogenesis of periodontal diseases. IL-1 beta have been demonstrated at increased levels in inflamed gingival tissues and gingival crevicular fluid. It is one of the most potent inducers of bone resorption and promotes connective tissue degradation through the induction of Matrix metalloproteinase (MMPs) gene expression.[6]

Pomegranate extract exhibited anti inflammatory activity through inhibition of NF-kB activity and prevention of ERK-1 and ERK-2 activation. Pomegranate extract have been shown to inhibit the IL-1β induced destruction of proteoglycan, expression of MMPs at the cellular level, NO and PGE_2 production.[7] Pomegranate therefore could be beneficial in treating periodontitis as it possess excellent anti-inflammatory effect.

Reactive oxygen species (ROS) plays an important role in tissue destruction when produced in excess. Antioxidants are groups of substances that are able to prevent the oxidation reaction of ROS, thereby offering protection. Currently, there is a growing interest in the linkage between antioxidants and periodontal disease. A significant antioxidant enzyme within mammalian tissues is Superoxide Dismutase, which catalyzes the dismutation of O_2^- to H_2O_2 and O_2. Superoxide Dismutase (SOD) has also been localized within the human periodontal ligament and may represent an important defense mechanism within gingival cells against superoxide release.[8]

The Superoxide Dismutase activity significantly improved following periodontal therapy, suggesting a positive response to nonsurgical periodontal therapy. Therefore, treatment of periodontal disease reduces oxidative stress by a concomitant reduction in inflammatory load by enhancing antioxidant levels. Pomegranate was found to exert potent antioxidant activity against lipid peroxidation.[9] The free radical scavenging activity of pomegranate depends on the polyphenols that involves electron donation to free radicals that converts them to relatively more stable compounds. Pomegranate extract exhibits inhibition of LPS induced oxidative stress by reducing ROS and NO generation and increasing Superoxide Dismutase 1 mRNA expression.

Pomegranate is rich in polyphenols such as Ellagitanins, Punicalagins, Punicallin and Gallotanin. The high antioxidant and anti inflammatory activity of pomegranate is due to high content of punicalagin.**[10]** Although various forms of pomegranate extract has been used, in our present study it is used as a gel form since they can deliver the drug to the site of disease activity at a required concentration and also can facilitate prolonged drug delivery.

Based on the anti-inflammatory and antioxidant properties of punicalagin, the present study is designed to analyse the levels of IL-1 beta and Superoxide Dismutase in GCF after 21 days of subgingival application with punicalagin gel, as an adjunct to Scaling and Root Planing in patients with Chronic Periodontitis.

The aim of the present study is

1. To evaluate the effects of subgingival application of punicalagin gelatin film as an adjunct to Scaling and Root Planing (SRP) compared with SRP alone in patients with Chronic Periodontitis.

2. To compare the changes in Probing Pocket Depth and Clinical Attachment Level gain following therapy.

3. To assess the anti-inflammatory and anti-oxidant effect of punicalagin gelatin film on IL-1β and SOD levels by ELISA method.

Chronic Periodontitis is an infectious disease, resulting in inflammation within the supporting tissues of the teeth, ultimately resulting in progressive attachment loss and bone loss and is characterized by periodontal pocket formation and/or recession of the gingiva. The goal of periodontal therapy is to get rid of the microbial etiology and control or eliminate disease progression. Apart from this, periodontal treatment should restore the lost form, function, esthetics and comfort. Scaling and Root Planning is the most widely used approach that effectively decreases the microbial load.

SCALING AND ROOT PLANING

Scaling and Root Planing, otherwise known as conventional, Non-Surgical Periodontal Therapy, is the process of removing or eliminating the etiologic agents – dental plaque, its products, and calculus – which cause inflammation, thus helping to establish a periodontium that is in remission of periodontal disease.

Tagge DL et al., (1975)[11] evaluated the response of soft tissue pockets to treatment by oral hygiene procedures alone or by oral hygiene procedures and root planning. The combination of oral hygiene and root planning resulted in a more significant clinical outcome when compared to oral hygiene procedure alone.

Lindhe J et al., (1982)[12] in a 24 month study compared the effects of Scaling and Root Planing to the Widman flap procedure. During the active treatment and subsequent 6 months, participants received a professional tooth cleaning every 2 weeks and prophylaxis every 3 months thereafter for the remainder of the study. It was found that Scaling and Root Planing procedures were almost as effective as the Widman flap procedure in preventing further attachment loss. The results also

showed that both Probing Depth reductions and gain in Clinical Attachment Levels were more pronounced in initially deep pockets than in an initially shallow pockets.

Christgau M et al., (2006)[13] evaluated the clinical and microbiological healing outcomes following Non Surgical Periodontal Therapy using a modified sonic scaler system versus Scaling and Root Planning with hand instruments. The study compared twenty Chronic Periodontitis patients and the clinical and microbiological parameters were assessed at baseline, 4 weeks and 6 months after treatment. The results showed that sonic scaler system and Scaling and Root Planning seem to provide similarly favourable periodontal healing results, although in deep pockets Scaling and Root Planning with hand instruments appeared to achieve a better resolution of inflammation.

Konopka L et al., (2012)[14] evaluated the effects of Scaling and Root Planing on levels of IL-1beta, IL-8 and MMP-8 in GCF in patients with periodontal disease. The results demonstrated that the short term non surgical therapy resulted in a significant improvement in periodontal indices and a marked decrease of IL-1beta, IL-8 and MMP-8 levels in gingival crevicular fluid.

Cabrera SM et al., (2015)[15] conducted a study to evaluate the effect of Scaling and Root Planing in Chronic Periodontitis patients by analyzing clinical, microbiological parameters and IL-1beta levels over a period of 12 months. The results demonstrated that there was a significant reduction of Porphyromonas gingivalis, Tanerella forsythia, Fusobacterium nucleatum and Aggregatibacter actinomycetemcomitans and this reduction was correlated with a decrease in clinical parameters as well as IL-1 beta reduction.

CYTOKINES

Cytokines act through receptors and are especially important in the immune system. Cytokines modulate the balance between humoral and cell-based immune responses and they regulate the maturation, growth, and responsiveness of particular cell populations. Some cytokines enhance or inhibit the action of other cytokines in complex ways.

Hansen HB et al., (1993)[16] demonstrated IL-1 as a powerful and potent bone resorbing cytokines. It has been found that IL-1α and IL-1β are equally important in stimulating bone resorption and probably exerts their effects on bone resorbing cells in several ways. It was shown that they stimulate the proliferation of precursor cells, but may also act indirectly on mature cells to stimulate bone resorption.

Faizuddin M et al., (2003)[17] evaluated the relationship between IL-1β levels in GCF and the inflammatory status of the periodontal tissues in the Indian population. Group 1 comprised of patients with clinically healthy gingiva with no loss of attachment. Group 2 comprised gingivitis patients with no attachment loss and Group 3 comprised gingivitis patients with attachment loss. The results demonstrated that the levels of IL-1β was significantly higher in group 3 compared to group 1 and group 2.

SUPEROXIDE DISMUTASE

Sukhtankar L et al., (2013)[18] analyzed the effects of Non Surgical Periodontal Therapy on Superoxide Dismutase levels in gingival tissues of Chronic Periodontitis patients. The gingival tissues were collected and the Superoxide

Dismutase activity was assessed at baseline and at 2 months after Non Surgical Periodontal Therapy. The results showed that NSPT improved the clinical parameters and restored the previously increased SOD levels to normal in Chronic Periodontitis patients.

Biju T et al., (2014)[19] estimated and compared the Superoxide Dismutase and Glutathione levels in serum of patients with Chronic Periodontitis, gingivitis and healthy gingival before and after non surgical periodontal therapy. There was significantly higher levels of Superoxide Dismutase and Glutathione found in healthy group when compared to other groups. Also it was reported that the post treatment level of Superoxide Dismutase was higher than the pretreatment levels in periodontitis and gingivitis groups.

SYSTEMIC DELIVERY OF ANTIBIOTICS

Systemic administration of antibiotics is a route of drug administration into the circulatory system so that the entire body is affected. Administration can take place via enteral administration (absorption of the drug through the gastrointestinal tract) or parenteral administration (generally injection, infusion, or implantation).

Kapoor A et al., (2012)[20] discussed the rationale, proper selection, dosage and the duration of the antibiotic therapy and mentioned about the various drawbacks of using systemic drug delivery. The drawbacks includes less blood concentration of the antibiotics, patient non compliance, antibiotic antagonism, inability of the antibiotic to penetrate to the site of infection, limited vascularity or decreased blood flow and unfavourable local factors such as decreased tissue pH or oxygen tension.

LOCAL DRUG DELIVERY

The local delivery of antimicrobial agents to periodontal pockets has the benefit of administering more drugs at the target site and the sustained release of antimicrobial in the periodontal pockets while minimizing the exposure of total body to the drug.

Several degradable and non-degradable devices are being used for the delivery of antimicrobial agents into the periodontal pocket including non-biodegradable fibres, films, bio-absorbable dental materials, biodegradable gels/ ointments, injectables, microcapsules and bioadhesive delivery systems that could significantly improve oral therapeutics for periodontal disease and mucosal lesions.

Singh G et al., (2014)[21] highlighted the indications and advantages regarding the use of local drug delivery systems. The indications were deep pockets with very difficult access for Scaling and Root Planning, Refractory Periodontitis patients, deep pockets that failed to respond to Scaling and Root Planning and medically compromised patients where surgical therapy is contraindicated. The advantages includes effective concentration at the site of delivery is achieved as 100 fold higher concentration is available at subgingival site when compared to systemic delivery. He also mentioned that superinfection and drug resistance are rare when drugs are given locally.

Ramesh A et al., (2016)[22] in a recent review discussed about various local drug delivery system and their effects. Based on the evidences, he indicated that Local Drug Delivery into periodontal pocket could improve the periodontal health. He also mentioned that controlled release properties can be applied as a therapeutic

component in the effective management of localized persisting lesion and the local drug administration should be based on patient clinical findings, scientific evidences and proper diagnosis.

MOUTHWASH

Alshehri M et al., (2015)[23] evaluated the effects of Scaling and Root Planing with and without essential oil based mouthwash on IgG levels in patients with periodontal inflammation. One group of patients underwent Scaling Root Planing followed by a 10 ml rinse of essential oil based mouthwash for 30 days. The other group received Scaling and Root Planning alone. It was seen that Scaling and Root Planning when used along with essential oil based mouthwash was more effective in reducing whole salivary IgG levels as compared to Scaling and Root Planing alone.

Nadkerny PV et al., (2017)[24] assessed and compared the antiplaque and anti-inflammatory effects potential of a probiotic mouthwash with 0.2% chlorhexidine and saline. The results depicted that both probiotic and chlorhexidine mouthwash lead to reduction of plaque Index, Gingival Index and OHI-S significantly. He also demonstrated that these mouthwash can be successfully used in prevention of plaque and gingivitis as an adjunct to mechanical plaque control methods.

FIBERS

Goodson JM et al., (1979)[25] tried to treat periodontal disease by placing drugs within the gingival sulcus. He used tetracycline filled hollow fibers in gingival sulcus and it had effect on both microbial count and clinical manifestation of the

disease. He also mentioned that 1/1000 of tetracycline needed for systemic therapy is sufficient for local delivery. He observed that elimination of spirochetes can be achieved by a single placement of tetracycline filled hollow fibers.

Grover HS et al., (2014)[26] compared two commercially available controlled release drugs – tetracycline fibers and chlorhexidine gel. Systemically healthy patients were chosen having Chronic Periodontitis. Three sites were involved having a probing depth of more than 6 mm. First site received tetracycline fibers, the second site with chlorhexidine gel and the third site was taken as a control with Scaling and Root Planing alone. The results demonstrated that there was statistically significant reduction in all clinical parameters such as Plaque Index, Bleeding Index, Probing pocket depth, Relative Attachment Level gain in tetracycline fibers and chlorhexidine gel site when compared to control site.

Polepalle T et al., (2017)[27] assessed the efficacy of local delivery of hyaluron 0.8% gel as an adjunct to Scaling and Root Planning in patients with Chronic Periodontitis. 0.2 ml hyaluron gel was placed subgingivally. Clinical parameters were assessed at 1st, 4th and 12th week. The results indicated that there was a significant reduction in Bleeding On Probing, Plaque Index, Probing Pocket Depth and Clinical Attachment Level in experimental site when compared to site that received Scaling and Root Planing alone.

GEL FORM

Akncbay H et al., (2007)[28] used Chitosan, a novel biodegradable natural polymer, in a gel form (1%, w/w) with or without 15% metronidazole adjunctive to Scaling and Root Planing in patients with Chronic Periodontitis. The clinical

parameters were assessed at 6, 12 and 24 months. The end results indicated that metronidazole gel delivered through chitosan gel can be effectively used in the treatment of Chronic Periodontitis.

STRIPS

Addy M et al., (1988)[29] evaluated the efficacy of chlorhexidine, Metronidazole and Tetracycline containing acrylic strips for the treatment of periodontal diseases. All the three antimicrobial agents differed on their action and spectra of activity. However all were found to have activity against many of the organisms considered pathogenic in periodontal diseases. Overall, the results demonstrated that subgingival antimicrobial drug in strip form have the potential value when used alone in treatment of Chronic Periodontal disease.

Takamori HYA et al., (1992)[30] developed a new controlled release film like strip containing Ofloxacin as an antibacterial agent. The drug was applied once a week for 4 weeks in periodontal pockets of Chronic Periodontitis patients. The results demonstrated that weekly application had a significant effect on the resolution of periodontal inflammation and has an inhibitory effect on supragingival plaque deposition.

Jeffcoat MK et al., (1998)[31] evaluated the efficacy of a controlled release biodegradable Chlorhexidine chip of 2.5 mg as an adjunct to Scaling and Root Planning. The chip released Chlorhexidine within the pocket over 7-10 days, maintaining an average concentration of Chlorhexidine in Gingival Crevicular Fluid greater than 125 microgram per ml for 8 days. At 9 months significant reductions from baseline clinical parameter value favouring the chlorhexidine chip compared

with both control treatments were observed with respect to probing depth and clinical attachment level. These datas demonstrate that the adjunctive use of the Chlorhexidine chip results in a significant reduction of Probing Depth when compared with both Scaling and Root Planing alone or the adjunctive use of a placebo chip.

Katiyar A et al., (2012)[32] conducted a study on periodontal film and demonstrated that the Lomefloxacin hydrochloride films can produce much higher levels of drug at the site of interest with no side effects in comparison to conventional oral therapy for prolonged periods.

Umadevi S et al., (2012)[33] demonstrated that Ciprofloxacin films had antibacterial activity on periodontal pathogens such as Porphyromonas gingivalis, Prevotella intermedia and Aggregatibacter actinomycetemcomitans at different concentrations.

MICROCAPSULES

Yeom HR et al., (1997)[34] evaluated the clinical and microbiological efficacy of subgingival delivery of 10% minocycline loaded microcapsules in 15 adult periodontitis patients. In the supragingival scaling + 10% Minocycline loaded group, spirochetes and motile rods decreased and the percent of cocci increased after 1 week. The results demonstrated that 10% Minocycline loaded microcapsules can reduce Probing Depth and Bleeding on probing greater than Scaling and Root Planning and can induce microbial response more favourable for periodontal health.

NATURAL HERBS

Herbal and natural products have been used for centuries in every culture throughout the world. Plants and natural products from time immemorial used for their pharmacological applications such as antiulcerogenic, wound healing, anti-inflammatory, antimicrobial, antioxidant properties etc.

Bonjar S et al., (2004)[35] reported 45 species of plants from which 29 plant families were used in the traditional medicine by the Iranian people and they had antibacterial activities against one or more of the bacterial species. He also depicted that those plant extracts were able to overcome the disadvantages of antibiotics like drug resistance and undesirable side effects.

Kala BS et al., (2015)[36] summarized the effects of natural products like Acaciacatechu, Aloe vera, Azadirachata indica, Ocimum sanctum, Punica granatum and other important herbs to treat various periodontal diseases. He concluded that the antimicrobial activities of these herbal drugs can be potentially used against periodontal diseases.

PUNICA GRANATUM

Tyagi S et al., (2012)[37] reported that punicalagins are tannins and are large phenolic compounds and are found in two forms α and β in pomegranates. He also mentioned that punicalagin represents major component responsible pomegranates antioxidant property. Punicalagins also have been shown to reduce cholesterol and lowers blood pressure and also have been reported to increase the speed at which heart blockages melts away.

Howell AB et al., (2013)[38] reported that methanolic extracts of the fruit, especially of the peel exhibited the broadest antibacterial activity. The methanolic extracts of pomegranate were found to be rich in hydrolysable tannins (Punicalins and Punicalagins), ellagic acid, a component of ellagitannins and gallic acid. He also added that pomegranate possess positive effects on probiotic bacteria.

Rawahi AS et al., (2013)[39] reported that polyphenols are widely distributed among many plants and contains more than 8000 phenolic compounds. Polyphenols forms major component of pomegranate and are known for their antioxidants, anti-inflammatory, antimutagenic, anticarcinogenic and antimicrobial property. He also reported that chromatographic analysis revealed the presence of over 61 total phenolic compounds which included Gallic acid, Ellagic acid, Punicalin, Punicallagin and Malic acid etc.

Sreekumar S et al., (2014)[40] in a review article has mentioned pomegranate extract as a rich source of biologically active compounds. The different parts of pomegranate plant like peel, root, bark, flower, leaves exhibited different phytochemicals. Punicalagin was found to be the main polyphenolic compound present in pomegranate peel. He also discussed about various therapeutic benefits and their mechanisms especially of antioxidants, anticarcinogenic and anti-inflammatory properties.

Prasad D et al., (2014)[41] has depicted that pomegranate juice possesses anti-atherosclerotic, anti-hypertensive, anti-aging and antioxidant effect. He also reported that the antibacterial property of pomegranate was due to the presence of tannins which increased bacteriolysis and interferes with bacterial adherens mechanisms onto the tooth. Pomegranate, thereby could serve as an excellent

adjunct to the conventional periodontal therapy as an anti-plaque agent due to its antibacterial properties.

Masaud IA et al., (2014)[42] reported that the high antioxidant capacity of pomegranate is largely attributed to the polyphenol Ellagitannins (Punicalagins and Punicalins). In this review, the chemotherapeutic potential of fruit extract against breast cancer, prostate cancer and colon cancer were considered and dictated that the anticancerous effect is due to the antioxidant capacity of pomegranate.

INVITRO STUDIES

Singh RP et al., (2002)[43] evaluated the efficacy of antioxidant rich fractions extracted from the peels and seeds of pomegranate. This was the first study on the antioxidant properties of extracts from pomegranate peel and seeds. The methanol extracts showed 56, 58 and 43.7 µg/ml inhibition using thiobarbituric acid method, hydroxyl radical scavenging activity and Low Density Lipoprotein oxidation respectively at 100 ppm.

Lee CJ et al., (2010)[44] evaluated 4 components of pomegranates for their anti-inflammatory property namely Punicalagin, Punicalin, Strictinin A and Granatin B. He demonstrated that all the four hydrolyzable tannins inhibited the NO production and iNOS expression in RAW 264.7 cells. The results suggested that all these 4 components can be used as a marker for anti-inflammatory effect of pomegranate.

Abdollahzadeh SH et al., (2011)[45] evaluated the effect of methanolic extract of Punica granatum peel against Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermis, Streptococcus salivarius, Lactobacillus

acidophilus and Candida albicans. Methanolic extract of Punica granatum peel were used at a concentration of 4mg/ml, 8mg/ml and 12mg/ml. The activity was evaluated by agar disk diffusion method. The results showed that all the concentrations inhibited the Staphylococcus epidermis and Staphylococcus aureus. 8mg/ml and 12 mg/ml were effective against Lactobacillus acidophilus, Streptococcus mutans and Steptococcus salivarius. The results suggested that pomegranate extract can be used as an antibacterial agent in controlling oral infections.

Xu X et al., (2014)[46] evaluated the mechanism behind the anti-inflammatory potential of punicalagin in LPS induced macrophages. The results indicated that PUN at concentrations of 25, 50 or 100 μM could significantly decrease the LPS induced NO, PGE_2, IL-1β, IL-6 and TNF-α in RAW 264.7 cells. Molecular research indicated that these effect were due to inhibition of NF-κβ via Iκβα and p-65 supression. The results concluded that Punicalagin can be a potential choice for the treatment of inflammatory diseases.

Aloqbi A et al., (2016)[47] evaluated the antioxidant activities of punicalagin and pomegranate juice. The antioxidant mechanisms included were DPPH radical scavenging activity, Hydrogen peroxide scavenging, Ferrous chelating and reducing ability. The results showed that punicalagin showed significant Ferrous chelating activity and reducing power ability in a dose dependent manner when compared to pomegranate juice.

INVIVO STUDIES

Murthy KNC et al., (2002)[48] tried to evaluate the antioxidant efficacy of methanolic extract of pomegranate on Catalase, Peroxidase and Superoxide Dismutase enzyme levels. He administered 50 mg/ml of methanolic extract of pomegranate followed by 2.08/kg of Ccl_4. Histopathological studies showed hepatoprotective effect of methanolic extract against Ccl_4. The results also showed that all the three antioxidant enzyme levels were preserved by the methanolic extracts.

Cerda B et al., (2003)[49] indicated the effects of punicalagins in rats and evaluated its possible toxic effect in Sprague – Dawley rats upon repeated oral administration of a 6% punicalagins containing diet for 37 days. The results reported that the high dose of punicalagin was non toxic to rats when administered even for 37 days.

Sastravaha et al., (2005)[50] evaluated the efficacy of combined herbal preparation in comparison to a standard Supportive Periodontal Therapy. Patients having Probing Pocket Depth of 5-8 mm after Conventional Periodontal Therapy were selected and given a medicated chip consisting of Centella asiatica and Punica granatum. The result showed improvement of Probing Depth, Attachment level, Gingival Index at 3 and 6 months and Bleeding Index at 6 months in test group compared to control group. Also the IL-1 level was significantly decreased in the test group.

Disilvestro RA et al., (2009)[51] compared the efficacy of pomegranate mouthwash against a placebo of cornstarch in water. It was a 4 week study in which

study population were advised to rinse the mouth with prescribed mouthwash thrice a day. The result demonstrated a reduction in total protein concentration, aspartate aminotransferase, alpha glucoside activity, increased antioxidant enzyme cerruloplasmin and increased radical scavenging activity. From the results it was concluded that pomegranate extracts can be included in oral health products.

Larrosa et al., (2009)[52] evaluated the beneficial effect of pomegranate against inflammatory markers. Male fischer rats were fed with 250mg/kg/day pomegranate extract. He noted that pomegranate extract decreased the inflammatory markers like iNOS, COX 2, PGE_2 in colonic mucosa and also modulated favourably the gut microbiota.

Bhadbhade SJ et al., (2011)[53] evaluated the antiplaque efficacy of pomegranate containing mouthwash. Patients were randomly divided into 3 groups and were given pomegranate, chlorhexidine and distilled water mouthwashes twice daily. Microbiological evaluation included Actinobacillus actinomycetemcomitans, Porphyromonas gingivallis and Prevotella intermedia. The results indicated reduction of Plaque index in all the three groups and reported that pomegranate mouthwash showed an increased antiplaque effect and was effective against Actinobacillus actinomycetemcomitans, Porphyromonas gingivallis and Prevotella intermedia at concentrations of 62.5mg/ml, 31.25mg/ml and 16.125mg/ml respectively.

Gomes et al., (2016)[54] evaluated the antibacterial effect of punica granatum extract against the periodontal pathogen P.gingivalis by using Galleria mellonella as an invivo model. Results showed that Punica granatum extract had antibacterial activity against P.gingivalis at all concentrations of 12.5, 6.25, 3.1 and 2.5 mg/ml

used in this study. The results also demonstrated that pomegranate extract can be used against periodontal pathogens.

Umar D et al., (2016)[55] evaluated the influence of pomegranate extract mouthrinse on salivary pH and the levels of Streptococcus mutans. The results showed a significant reduction in the Streptococcus mutans count on usage of pomegranate extract mouth rinse. Therefore the present study implies that pomegranate extract can be used to maintain good oral hygiene and as an antibacterial compound.

A randomized controlled, split mouth clinical study was conducted to evaluate the effectiveness of subgingival application of punicalagin gelatin film as an adjunct to Scaling and Root Planning compared with scaling and root planning alone in patients with chronic periodontitis. The protocol was reviewed and approved by institutional ethical board. The study related procedures were explained to the patients before they sign an informed consent form. A total of 30 subjects each with bilateral 5-6 mm probing pocket depth (PPD) were recruited from the outpatient in Department of Periodontics, J.K.K. Nattraja Dental College and Hospitals, Kumarapalayam, Tamilnadu.

Inclusion criteria

1. Patient age group of 20-50 years with generalised chronic periodontitis
2. Minimum of 20 teeth to be present in every patient
3. Minimum of 2 sites with more than 5mm of pocket depth as assessed by Williams periodontal probe
4. Minimum of 6 sites which shows bleeding on probing

Exclusion criteria

1. Patients suffering from known systemic diseases
2. Patients who are pregnant and lactating
3. Patients who are smokers and alcoholics
4. Patients who received any chemotherapeutic mouth rinse or oral irrigation during past 6 months
5. Patients who received surgical or non surgical therapy in last 6 months

6. Patients with Aggressive Periodontitis

7. Patients who received any antibiotic therapy in the last 6 months

STUDY DESIGN

A split mouth design was followed, where two sites with probing pocket depth of 5-6 mm were chosen. Probing Pocket Depth standardization was done with acrylic stent in all the selected areas.

CRITERIA FOR GROUPING

Selected sites were randomly divided into control sites and experimental sites as follows

- **Group I -** It consists of 30 sites, in which Scaling and Root Planning (SRP) was done (control sites).
- **Group II -** It consists of 30 sites, in which scaling and root planning was followed by the placement of the punicalagin gelatin film inside the pocket (SRP + Punicalagin gelatin film) (Test sites).

CLINICAL PARAMETERS

The following variables were measured at baseline and at 21 days

- Plaque index (PI) (Silness and Loe 1964)
- Gingival index (GI) (Loe and Silness 1963)
- Sulcus Bleeding Index (SBI) (Muhlemann HR and Sen S 1971)
- Probing Pocket Depth (PPD)
- Clinical attachment level (CAL)

1. Plaque index (Silness and Loe 1964)

The four gingival areas of the tooth surfaces examined are the disto-facial, facial, mesio-facial and lingual surfaces.

Scoring was as follows:

0 – No plaque.

1 – A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen only by running a probe across the tooth surface.

2 – Moderate accumulation of soft deposits within the gingival pocket, on the gingival margin and/or adjacent tooth surface, which can be seen by the naked eye.

3 – Abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface.

The scores of the four areas of the tooth can be summed and divided by four to give the PI for the tooth. A score from

0.1– 0.9 – Good.

1.0 – 1.9 – Fair.

2.0– 3.0 – Poor

2. Gingival index (Loe and Silness 1963)

The Gingival index was created to assess the severity of gingivitis based on its color, consisitency and bleeding on probing. The tissues surrounding each tooth are divided into four gingival scoring units: Distal facial papillae, Facial margin, Mesial facial papillae, Entire lingual gingival margin.

The criteria are

0 – Normal gingiva

1 – Mild inflammation – slight change in color and slight oedema but no bleeding on probing.

2 – Moderate inflammation – redness, oedema and bleeding on probing.

3 – Severe inflammation – marked redness and oedema, ulceration with tendency to spontaneous bleeding.

The scores of four areas of the tooth can be summed and divided by four to give the GI for the tooth. A score from.

0.1– 1.0 – Mild inflammation

1.1– 2.0 – Moderate inflammation

2.1 – 3.0 – Severe inflammation

3. Sulcus Bleeding Index (SBI) (Muhlemann HR and Sen S 1971)

An early sign of gingivitis is bleeding on probing and, in 1971, Muhlemann and Son described the Sulcus Bleeding Index (SBI). The criteria for scoring are as follows:

Score 0 – health looking papillary and marginal gingiva no bleeding on probing;

Score 1 – healthy looking gingiva, bleeding on probing;

Score 2 – bleeding on probing, change in color, no edema;

Score 3 – bleeding on probing, change in color, slight edema;

Score 4 –bleeding on probing, change in color, obvious edema;

Score 5 –spontaneous bleeding, change in color, marked edema.

Four gingival units are scored systematically for each tooth: the labial and lingual marginal gingival (M units) and the mesial and distal papillary gingival (P units). Scores for these units are added and divided by four. Adding the scores of the undivided teeth and dividing them by the number of teeth can determine the sulcus bleeding index.

The least score is 0. The maximum score is 320.

4. Probing pocket depth (PPD) was recorded by selected sites using William's graduated periodontal probe. The probe was inserted parallel to the long axis of the tooth gently, until resistance was felt and the readings were recorded to the nearest millimeter from the gingival margin to the base of the pocket. Acrylic stents were used to standardize the path of insertion and angulations of the probe.

5. Clinical attachment level (CAL) - Distance between the base of the pocket and Cementoenamel Junction (CEJ) or a fixed reference point. The distance from the CEJ (if CEJ not detected, the coronal border of the stent was used) to the base of the pocket was measured.The readings were recorded to the nearest millimeter.

INVITRO STUDIES

Preparation of Test Organism

Preparation of standard bacterial suspensions

The average number of viable, Gram negative Porphyromonas gingivalis organisms per ml of the stock suspensions was determined by means of the surface viable counting technique (Miles and Misra, 1938). About (10^5-10^6) colony-forming

units per ml was used. Each time, a fresh stock suspension was prepared; the experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

Antibacterial Activity Screening

Determination of the Minimal Inhibitory Concentrations (MIC)

MIC was determined with 96-well plate microdilution method. Briefly, the bacterial strain was grown for 24 h anaerobically and inoculated into a final volume of 100 μL of new half-strength BHI broth containing 2-fold serial dilutions of samples. The final optical density of the bacterial cells was adjusted to 0.1 at 600 nm in 100 μL of mixture. The mixture was cultured anaerobically at 37 °C for 48 hour. The bacterial growth was measured at an optical density of 600 nm. The lowest concentration at which no growth (OD 600nm ≤ 0.1) was observed was defined as MIC (μg/mL). The concentrations of the sample used for MIC were between 100-7.25 μg/mL.

Preparation of PC periodontal films

Materials : Punicalagin (PC), Carbosynth Ltd, United Kingdom. Gelatin, Sterling Biotech Limited, Tamilnadu, India. Glycerine and Triethanolamine SD Fine chemicals, Mumbai, India. Sorenson's phosphate buffer pH 6.6

Methods :

Film formulations were prepared using the solvent casting technique (Table 1). Gelatin solution (20%) was prepared by dissolving gelatin in Millipore water. 1% v/v glycerol, which was used as a plasticizer to impart adequate flexibility to the

produced films. Calculated amount of PC (10mg/film) was added to the final gel before casting. The pH of gel formulation was adjusted to about neutrality (pH 6) by the addition of triethanolamine (0.3%) before casting. The medicated gels were left overnight at room temperature to get rid of any bubbles formed while stirring. The gels were casted into square cube trays and allowed to dry in a levelled oven maintained at 30^{o}C, for a period of time enough to produce flexible, dry film with constant weight. The dried films were cut into rectangular (5X2 mm) patches, packed in aluminum foil and stored in desicator which was maintained at room temperature.

Composition of PC periodontal films

Composition	**Concentration (%)**
PC	10mg/film
Gelatin	20
Glycerine	3
Triethanolamine	0.3

Physical characterization and content uniformity test

Assessment of weight and thickness was done on six randomly chosen film patches from each formulation using a sensitive balance (Electronic balance, Sartorius AG, weighting technology, BL-210S, Germany) and a digital micrometer (Tricircle micrometer, China), respectively. Determinations were performed in triplicate. Drug content uniformity was tested on six randomly selected film patches of each formulation. Each drug-loaded patch was allowed to dissolve in 100 ml of Sorenson's phosphate buffer pH 6.6. The concentration of PC in the patch was determined spectrophotometrically at 378 nm. UV standard curves were constructed

over a concentration range of 5-25 mg/ml. All results are presented as mean ± standard deviation.

Surface pH

Film patches were allowed to swell for 2 hr on the surface of agar plates prepared in Sorenson's phosphate buffer pH 6.6. The surface pH was determined using pH paper placed on the surface of the swollen patch. A mean of three readings were recorded.

In vitro release study

Each disc (5X2 mm) was weighed and placed into a 5 ml vial containing 2 ml Sorenson buffer of pH 6.6, previously warmed at 37^{o}C. The closed vials were placed in a thermostatically controlled water bath preset at 37^{o}C, until the end of the experiment. The whole volume was withdrawn at predetermined time intervals (0.5, 1, 2, 3, 4, 6, 8, 24, 48 and 72 h) and replaced by fresh warmed buffer solution. The samples were assayed for PC spectrophotometrically at λmax 378 nm, and the cumulative drug concentrations were calculated. All experiments were done in triplicate and the values were presented as the mean ± standard deviation. Blank films were also subjected to the release study to detect the contribution of the polymers used, if any, to UV absorption.

Calibration curve

From 100 mg/mL of stock solution a series of dilutions ranging from 5 - 25 mg/ml was prepared. The sample was analysed through UV-Vis spectrophotometer at a λ max of 378 nm and the calibration curve was plotted.

In vitro release study

A burst release of PC from both types of films was observed throughout the first 2 hr. This effect was followed by a decrease in the release rate for the next 10 h, then by a marked decrease in rate to the end of the study. Release of PC from gelatin films was performed in 2 ml buffered system at pH 6.6 to simulate the small space available of the periodontal cavity, the un-sink condition and pH. The initial burst release effect observed from all films could be explained by the fact that the drug may exist in the finely divided state after solvent evaporation during film casting and their deposition on the surface of the films after drying. Two main targets are to be achieved when inserting a medicated film in the periodontal pocket, to release an initial high dose of drug in order to produce an immediate therapeutic effect, followed by small doses to maintain the therapeutic level throughout a longer period of time. The general initial increase of the dissolution of PC from gelatin films could also be attributed to the presence of the water-soluble gelatin and also water-soluble hydrophilic additives in these films that would dissolve rapidly introducing porosity. The formed voids will in turn allow for the entrance of the release media and its diffusion through the film.

BIO-CHEMICAL PARAMETERS

Collection of preoperative GCF

The test and control sites were isolated by cotton rolls and gently air-dried to remove saliva. Before gingival crevicular fluid collection supragingival plaque was removed. A 2 µL sterile glass microcapillary tube was placed at the opening of the periodontal pocket and left for 30 seconds to draw 0.25 µL of gingival crevicular

fluid (draw 4 mm along 32 mm tube, holding maximum volume of 2 µL) into the microcapillary tube.

The sample was discarded if blood was detected within the microcapillary tube. Each gingival crevicular fluid sample was immediately placed into a sterile, labeled Eppendorf tube and placed on ice then transported to the laboratory for processing.

Non-Surgical Periodontal Therapy

At baseline full mouth ultrasonic scaling was done. Under local anesthesia with 2% lignocaine solution (1:80,000), root planning was done in the test and control sites using area-specific double-ended Gracey curettes (Hu-Friedy). The test site was isolated with cotton rolls to prevent contamination with saliva. The Punicalagin gel was carried with a tweezer and placed in the periodontal pocket. The pocket opening was covered with Coe-Pak to retain the material in the pocket, as well as to prevent the ingress of oral fluids.

Oral hygiene maintenance instructions were given as in Appendix I. Subjects were recalled at 7^{th} and 14^{th} day for application of punicalagin gelatin film and after 21 days post operative GCF samples were collected. Clinical parameters were repeated after 21 days.

Determination of IL-1β level in GCF using ELISA

Level of IL-1β in the GCF was assayed by using commercially available Human IL-1 beta ELISA reagent set (e-Bioscience).

Description

This Human IL-1 beta ELISA reagent set (with or without high-affinity binding microwell plates) contains the necessary reagents, buffers and diluents for performing quantitative enzyme linked immune sorbent assays (ELISA). This ELISA reagent set is specifically engineered for accurate and precise measurement of human IL-1 beta protein levels from samples including serum, plasma, and supernatants from cell cultures. This ELISA will recognize the cleaved mature form and uncleaved pro-form of Human IL-1 beta.

Reagents used

- Capture Antibody**:** Pre-titrated, purified antibody.
- Detection Antibody: Pre-titrated, biotin-conjugated antibody.
- Standard Recombinant cytokine for generating standard curve and calibrating samples
- ELISA/ELISPOT Coating Buffer Powder: This ELISA Set may contain ELISA/ELISPOT Coating Buffer Powder (Reconstitute to 1L with dH20 and filter (0.22 uM)) or 10X PBS ELISA Coating Buffer (Dilute 1 part 10X Buffer into 9 parts dH20).
- Assay Diluent: 5X concentrated
- Detection enzyme: Pre-titrated Avidin-HRP
- Substrate Solution: Tetramethylbenzidine (TMB) Substrate Solution
- 96 Well Plates

Experimental Procedure

Coat Corning Costar 9018 (or Nunc Maxisorp) ELISA plate with 100 µL/well of capture antibody in Coating Buffer (dilute as noted on C of A, which is included with the reagent set). Seal the plate and incubate overnight at 4°C. Aspirate wells and wash 3 times with >250 µL/well Wash Buffer. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer.

Dilute 1 part 5X concentrated Assay Diluent with 4 parts DI water. Block wells with 200 µL/well of 1X Assay Diluent. Incubate at room temperature for 1 hour wells. Perform 2-fold serial dilutions of the top standards to make the standard curve for a total of 8 points. Add 100 µL/well of your samples to the appropriate wells. Seal the plate and incubate at room temperature for 2 hours (or overnight at 4°C for maximal sensitivity).

Aspirate/wash as in step 2. Repeat for a total of 3-5 washes. Add 100 µL/well of detection antibody diluted in 1X Assay Diluent. Seal the plate and incubate at room temperature for 1 hour. Aspirate/wash as in step 2. Repeat for a total of 3-5 washes. Add 100 µL/well of Avidin-HRP diluted in 1X Assay Diluent. Seal the plate and incubate at room temperature for 30 minutes.

Aspirate and wash as in step 2. In this wash step, soak wells in Wash Buffer for 1 to 2 minutes prior to aspiration. Repeat for a total of 5-7 washes. Add 100 µL/well of Substrate Solution to each well. Incubate plate at room temperature for 15 minutes.

Add 50 µL of Stop Solution to each well. Read plate at 450 nm. If wavelength subtraction is available, subtract the values of 570 nm from those of 450 nm and analyze data.

Interpretation of Results

Sample results were expressed as pg/mL and do not need to be corrected for dilution (unless sample was diluted prior to testing).

ENZYME LINKED IMMUNOSORBENT ASSAY:

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of superoxide dismutase in human serum, plasma, tissue homogenates, cell lysates and other biological fluids. An anti-human SOD coating antibody is adsorbed onto microwells. Human SOD present in the sample or standard binds to antibodies adsorbed to the microwells. A HRP-conjugated anti-human SOD antibody is added and binds to human SOD captured by the first antibody. Following incubation unbound HRP- conjugated anti-human SOD is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of human SOD present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human SOD standard dilutions and human SOD concentration determined.

REAGENTS PROVIDED WITH KIT

Reagents for human SOD ELISA ALX-850-033

a) 1 Aluminium pouch with a Microwell Plate coated with monoclonal antibody to human SOD.
b) 1 Vials (20 µl) HRP-Conjugate anti-human SOD monoclonal antibody
c) 2 vials (500 µl) human SOD Standard, 5 ng/ml

d) 1 vial (5 ml) Assay Buffer Concentrate 20x (PBS with 1% Tween 20 and 10% BSA)

e) 1 vial (5 ml) Phosphate Buffered Saline Concentrate (PBS) 20x

f) 1 bottle (50 ml) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)

g) 1 vial (15 ml) Substrate Solution (tetramethyl - benzidine)

h) 1 vial (15 ml) Stop Solution (1M Phosphoric acid)

i) 1 vial (0.4 ml) Blue-Dye

j) 1 vial (0.4 ml) Green-Dye

k) 2 Adhesive Films

MATERIALS REQUIRED BUT NOT AVAILABLE WITH THE KIT:

1. 5 ml and 10 ml graduated pipettes

2. 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips

3. 50 µl to 300 µl adjustable multichannel micropipette with disposable tips

4. Multichannel micropipette reservoir

5. Beakers, flasks, cylinders necessary for preparation of reagents

6. Device for delivery of wash solution (multichannel wash bottle or automatic wash system)

7. Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)

8. Glass-distilled or deionized water

9. Statistical calculator with program to perform regression analysis

ASSAY PROCEDURE:

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

Wash the microwell strips twice with approximately 400 µl WashBuffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

Add 100 µl of PBS (1x) in duplicate to standard wells B1/2- G1/2, leaving A1/A2 empty. Pipette 200 µl of undiluted standard (concentration = 5.00 ng/ml) in duplicate into well A1 and A2. Transfer 100 µl to wells B1 and B2. Mix the contents of wells B1 and B2 by repeated aspiration and ejection, and transfer 100 µl to wells C1 and C2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of humanSOD standard dilutions ranging from 5.00 to 0.08 ng/ml. Discard 100 µl of the contents from the last.

Add 100 µl of PBS (1x)in duplicate to the blank wells.

Add 90 µl of PBS (1x)to the sample wells.

Add 10 µl of each prediluted sample in duplicate to the sample wells.

Prepare HRP-Conjugate.

Add 50 µl of HRP-Conjugate to all wells.

Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, if available on a microplate shaker set at 100 rpm.

Remove adhesive film and empty wells. Wash microwell strips 3 times according to point 0. of the test protocol. Proceed immediately to the next step.

Pipette 100 µl of TMB Substrate Solution to all wells.

Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

Stop the enzyme reaction by quickly pipetting 100 µl of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be

read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2-8°c in the dark.

Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

APPENDIX-1

POST THERAPY INSTRUCTIONS

- Report immediately on development of any untoward reactions like pain, swelling, bleeding and drug allergies.
- Avoid intake of any hard and hot foods, not to disturb the operated area with tongue.
- Report if dressing is dislodged.
- Avoid brushing the treated area from the day of therapy. Use cotton applicator to gently clean the area and resume gentle brushing with soft brush.
- Avoid the use of medications and mouthrinses.
- Follow up visits have to be done in 7^{th} day, 14^{th} day and 21^{st} days .

APPENDIX - 2

PROFORMA

NAME: **O.P**

NUMBER:

AGE: **DATE:**

SEX: **CASE**

NO:

ADDRESS:

OCCUPATION:

CHIEF COMPLAINT:

DENTAL HISTORY:

MEDICAL HISTORY:

PERSONAL HISTORY:

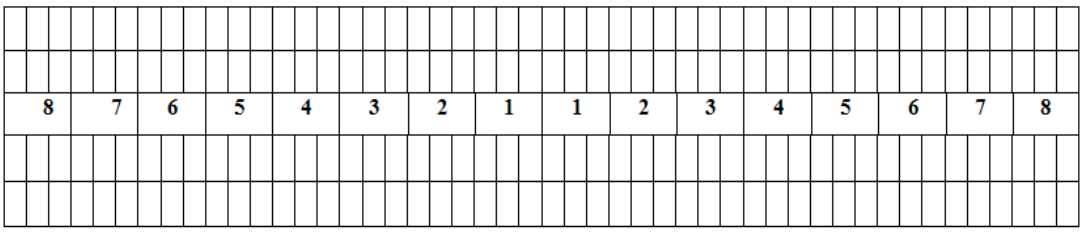
TEST SITE :

CONTROL SITE:

CLINICAL PARAMETERS

AT BASELINE

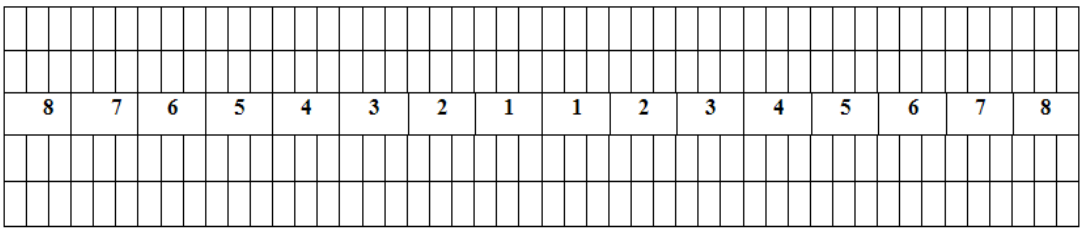
PLAQUE INDEX



Score ☐

Interpretation

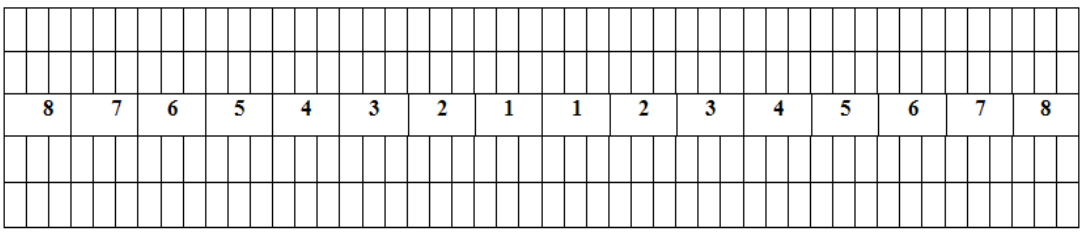
GINGIVAL INDEX



Score ☐

Interpretation

SULCUS BLEEDING INDEX



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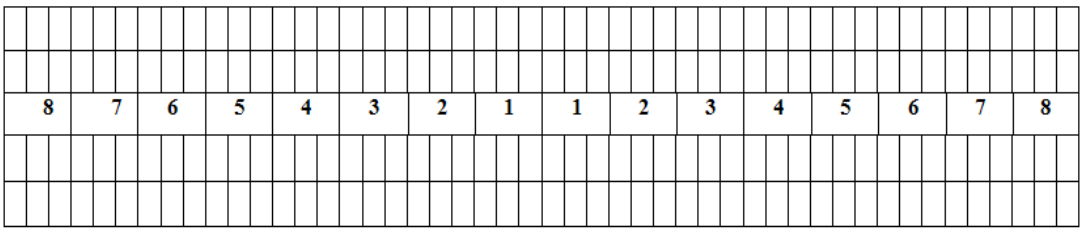
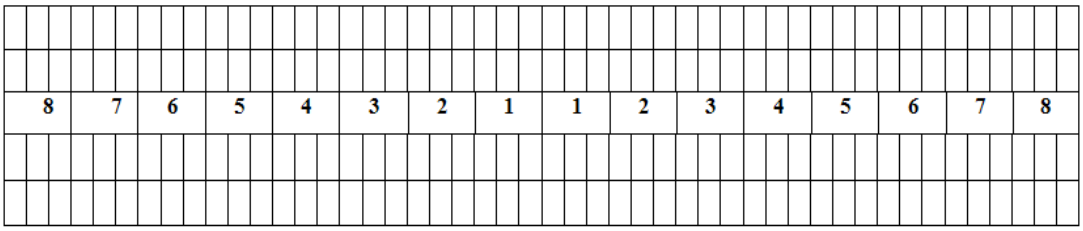
Interpretation:

PROBING POCKET DEPTH(mm)

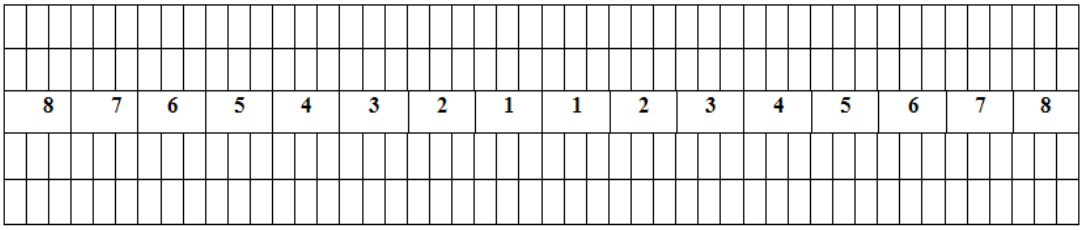


CLINICAL ATTACHMENT LEVEL(mm)



AT 3 WEEKS

PLAQUE INDEX



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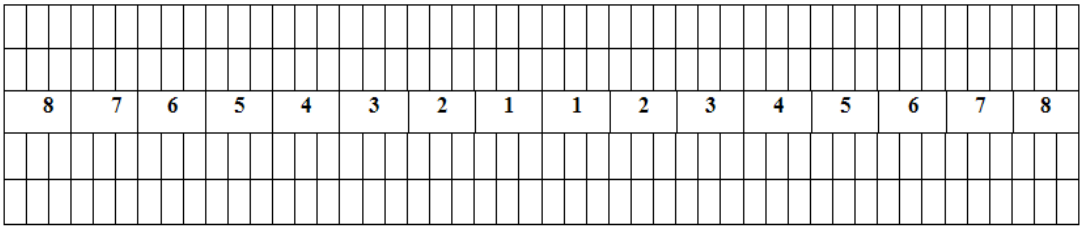
Interpretation

GINGIVAL INDEX



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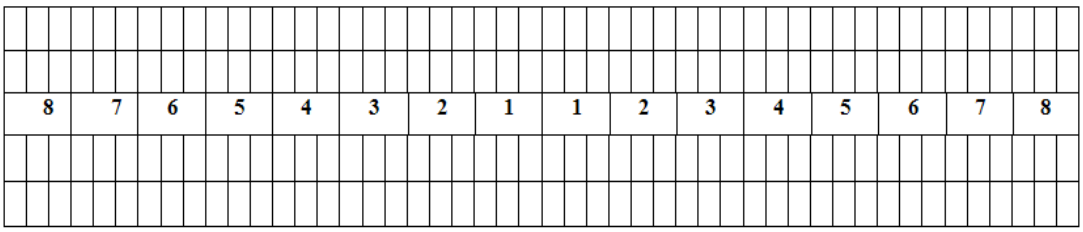
Interpretation

SULCUS BLEEDING INDEX



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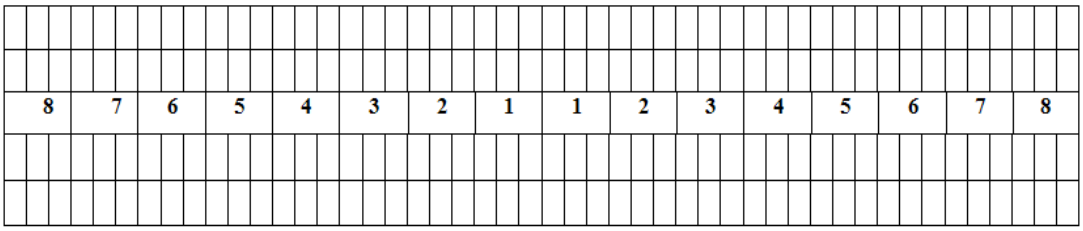
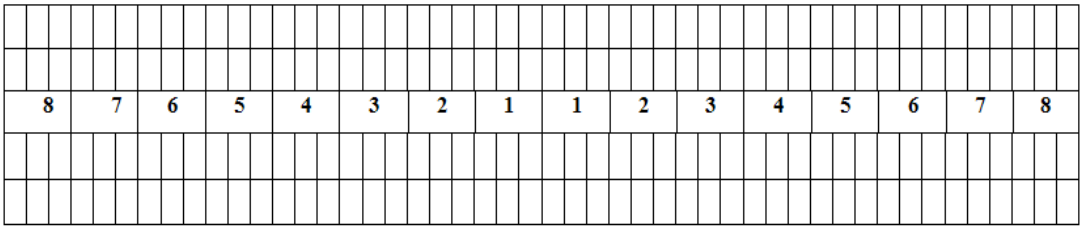
Interpretation:

PROBING POCKET DEPTH(mm)



CLINICAL ATTACHMENT LEVEL(mm)



J.K.K. NATTRAJA DENTAL COLLEGE, KOMARAPALAYAM

DEPARTMENT OF PERIODONTICS

INFORMED CONSENT OBTAINED FROM THE PATIENT

PATIENT NAME: _____

I have been explained about the nature and purpose of the study in which, I have been asked to participate. I understand that I am free to withdraw my consent and discontinue at any time without prejudice to me or effect on my treatment.

I have been given the opportunity to question about the material and study. I have also given the consent for photographs to be taken at the beginning, during and end of the study. I agree to participate in this study.

I hereby give the consent to be included in this study.

Place:

Date: **Signature of Patient**

APPENDIX – 3

ARMAMENTARIUM

MATERIALS AND INSTRUMENTS USED FOR THE STUDY

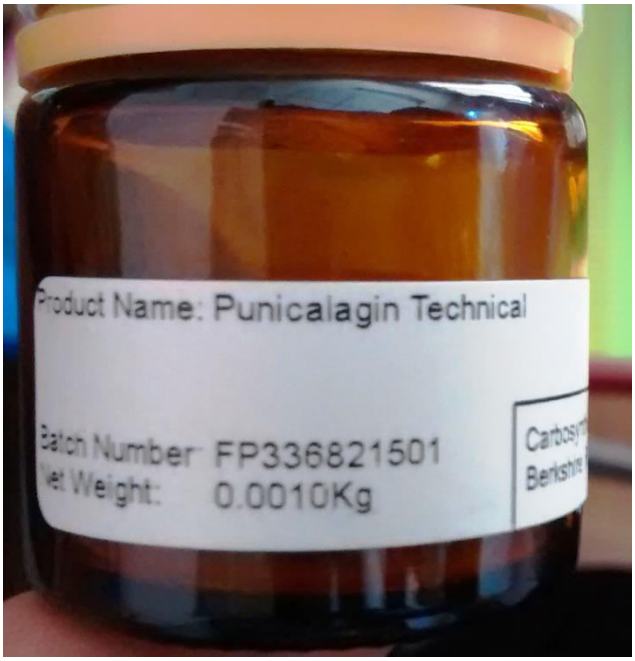
- Gloves
- Mouth mask
- Patient apron
- Chair apron
- Head cap
- Sterile cotton rolls
- Gauze
- Saline
- Kidney tray
- Betadine
- Lignocaine
- Syringe
- Mouth mirror
- Straight probe
- Explorer

- Williams periodontal probe
- Tweezer
- Tissue holding forceps
- Hu-Friedy Gracey Curettes
- Microcapillary pipettes
- Eppendorf tubes
- Phosphate buffered saline
- Punicalagin gelatin film
- Scissor
- Coe pak

URGICAL INSTRUMENTS

MICROCAPILLARY PIPETTES, EPPENDORF TUBES

PUNICALAGIN POWDER



PUNICALAGIN GELATIN FILM

CLINICAL CASES

PRE-OPERATIVE

CONTROL GROUP

TEST GROUP

GCF SAMPLE COLLECTION

PLAQUE SAMPLE COLLECTION

SRP (SCALING AND ROOT PLANING)

CONTROL GROUP

TEST GROUP

TEST GROUP

SRP + PUNICALAGIN GELATIN FILM PLACED

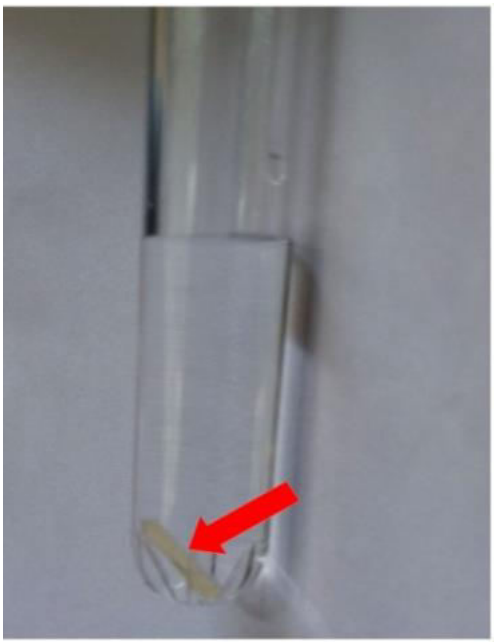
COE PACK PLACEMENT

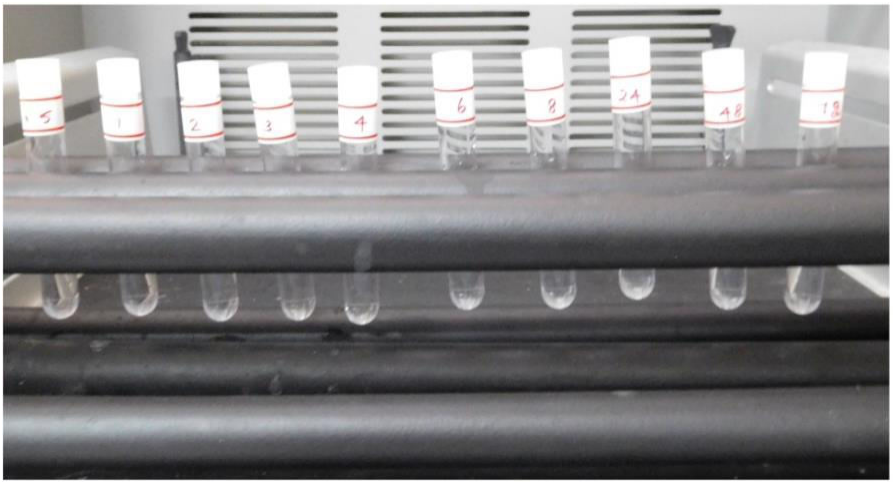
POST OPERATIVE (21 DAYS)

CONTROL GROUP

TEST GROUP

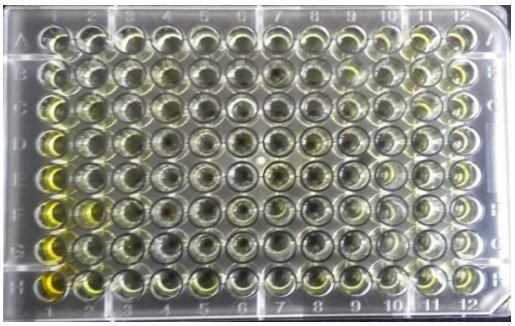
CUMULATIVE RELEASE PROFILE OF PC FROM GELATIN IN BUFFER PH 6.6.

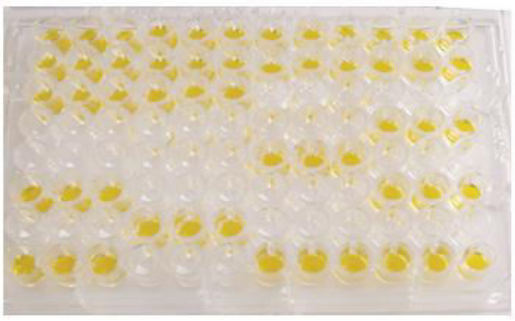




LAB ANALYSIS

ELISA KIT





STATISTICAL ANALYSIS

The results obtained were analyzed statistically and comparisons were made between groups using Paired samples t test. 'p value' between Group I and Group II post-operatively were evaluated. $p<0.001$ denoted statistically significant and also denotes less than one in a thousand chance of being wrong. $p<0.05$ denotes that there is only 5% chance that the null hypothesis is true. The statistical analysis was done using SPSS software Version 19.

Clinical evaluation

A total of 30 subjects were selected for the study and were divided in a split mouth design. Group I subjects were treated with SRP alone, whereas in Group II subjects local delivery of punicalagin gel along with SRP was done. No patient reported any discomfort or adverse reaction.

Plaque index (PI)

The mean Plaque Index score at baseline was found to be 2.37 ± 0.62. After 21 days of treatment the value was reduced to 1.39 ± 0.58 as shown in table 3 and graph 4. Statistically significant difference was found from baseline to 21 days post-treatment ($p<0.001$).

Gingival Index (GI)

At baseline, the mean Gingival Index score was 2.53 ± 0.67, and was reduced to 1.43 ± 0.52 after 21 days post-treatment as shown in table 3 and graph 4. There was statistically significant reduction in gingival index after 21 days post-treatment ($p<0.001$).

Sulcus Bleeding Index (SBI)

SBI at baseline was 2.75 ± 0.28 and was decreased to 1.75 ± 0.29 after 21 days post-therapy as shown in table 3 and graph 4. There was statistically significant decrease in bleeding after 21 days post-therapy ($p<0.001$).

Probing pocket depth (PPD)

In Group I the mean PPD at baseline was 5.69 ± 0.00 mm and after 21 days of post-treatment the value reduced to 2.92 ± 0.17 mm. In Group II, at baseline the mean PPD was 5.74 ± 0.00 mm, and at 21 days reduced to 2.05 ± 0.62 mm as shown in table 4 and graph 5. There was statistically significant reduction in PPD after 21 days post-therapy in both groups ($p<0.001$). Group II showed more statistically significant reduction in PPD at 21 days post-therapy, compared to group I ($p<0.001$) as shown in table and graph. 15.6% more reduction of PPD was seen in group II (64.3%) when compared to group I (48.7%) as shown in table 5 and graph 7.

Clinical Attachment Level

In Group I, at baseline the mean CAL was 3.69 ± 0.22 mm and at 21 days reduced to 1.82 ± 0.48 mm. In Group II, at baseline the mean CAL was 3.74 ± 0.38 mm and at 21 days reduced to 0.82 ± 0.22 mm as shown in table 6 and graph 6. There was statistically significant gain in CAL after 21 days post-therapy in group II ($p<0.001$). Group II showed more statistically significant gain in CAL at 21 days post-therapy, compared to group I ($p<0.001$) as shown in table 6 and graph 6. 27.6% more gain in CAL was seen in group II (79.1%) when compared to group I (51.5%) as shown in table 7 and graph 8.

IL-1β level in GCF

At baseline, the mean level of IL-1β in GCF in Group I was 78.23 ± 0.36 pg/ml and decreased to 59.53 ± 0.22 pg/mL 21 days after therapy. In Group II, the baseline value was 79.36 ± 0.18 pg/ml and decreased to 37.9 ± 0.83 pg/ml after 21 days post-therapy as shown in table 8. There was statistically significant decrease in the level of IL-1β after 21 days post-therapy in both the groups ($p<0.001$). When comparing both the groups, Group II has more statistically significant ($p<0.05$) decrease in the level of IL-1β than Group I post-therapy as shown in graph 9.

SOD Levels

In Group I, at baseline the superoxide dismutase levels were 111.83 ± 0.97 U/0.5ml and 21 days after treatment SOD levels improved to 189.73 ± 0.27 U/0.5ml. Comparison of SOD levels at baseline and after treatment shows a statistically significant difference with $p<0.05$. In Group II, the superoxide dismutase levels at baseline was 124.56 ± 0.74 U/0.5ml and 21 days after treatment SOD levels improved to 263.36 ± 0.23 U/0.5ml. Comparison of SOD levels at baseline and after treatment shows that there is a statistically significant difference with $p<0.05$. When comparing both the groups, Group II has more statistically significant ($p<0.05$) increase in the level of SOD than Group I after 21 days post-therapy as shown in table 9 and graph 10.

TABLE 1 : Composition of PC periodontal films

Composition	**Concentration (%)**
PC	10mg/film
Gelatin	20
Glycerine	3
Triethanolamine	0.3

TABLE 2 : Physical characteristics of the prepared films

Formulation	**Weight (mg±SD)**	**Thickness (mm ± SD)**	**Surface pH**	**Drug content (mg)**
PC Gelatin film	28.47 ± 3.13	2.13 ± 0.012	5.8-6	9.6 ± 0.8

*Results are presented as mean ± SD, n = 3.

TABLE 3 : Mean Gingival index, Plaque Index and Sulcus Bleeding Index at baseline and 21 days

Indices	**Baseline**	**21 days**	**p – value**
Gingival index	2.53 ± 0.67	1.43 ± 0.52	< 0.001*
Plaque index	2.37 ± 0.62	1.39 ± 0.58	< 0.001*
Sulcus Bleeding Index	2.75 ± 0.28	1.75 ± 0.29	< 0.001*

TABLE 4 : Comparison of mean in PPD between Group I and Group II post-therapy.

Probing pocket depth	**Group I** Mean±SD	**Group II** Mean±SD	**P Value**
Baseline	5.69 ± 0.00	5.74 ± 0.00	–
21 days	2.92 ± 0.17	2.05 ± 0.62	< 0.001*

TABLE 5 : Comparison of % of reduction in PPD between Group I and Group II post-therapy.

PROBING POCKET DEPTH	GROUP I	GROUP II	P VALUE
% Reduction	48.7%	64.3%	< 0.001*

TABLE 6 : Comparison of mean in CAL between Group I and Group II post-therapy

CLINICAL ATTACHMENT LEVEL	**Group I** Mean±SD	**Group II** Mean±SD	**P Value**
Baseline	3.69 ± 0.22	3.74 ± 0.38	–
21 days	1.82 ± 0.48	0.82 ± 0.22	< 0.001*

TABLE 7 : Comparison of % of gain in CAL between Group I and Group II post-therapy

CLINICAL ATTACHMENT LEVEL	GROUP I	GROUP II	P VALUE
% GAIN	51.5%	79.1%	< 0.001*

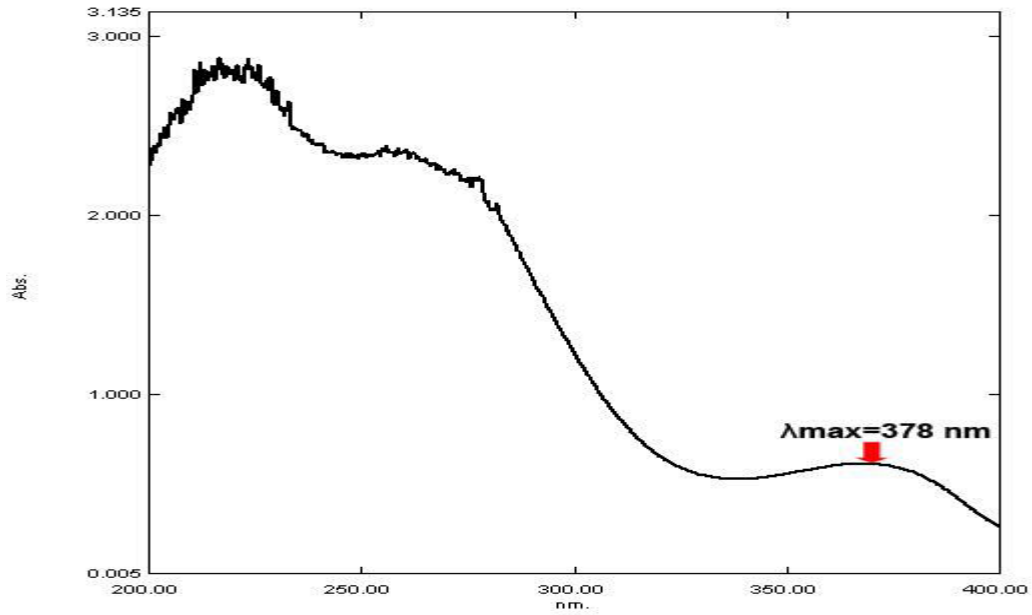
TABLE 8 : Comparison of IL-1β levels between group I and group II at baseline and at 21 days post therapy

IL-1β	GROUP I	GROUP II	P VALUE
BASELINE	78.23 ± 0.36	79.36 ± 0.18	_
21 Days	59.53 ± 0.22	37.9 ± 0.83	< 0.05*

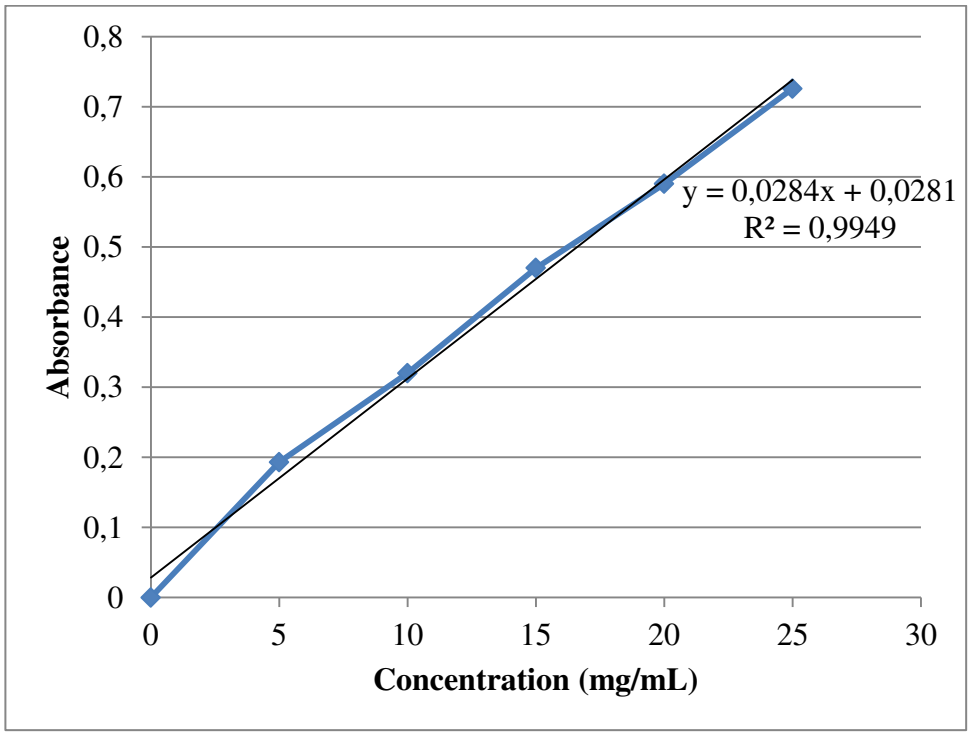
TABLE 9 : Comparison of SOD levels between group I and group II at baseline and at 21 days post therapy

SOD	GROUP I	GROUP II	P VALUE
BASELINE	111.83 ± 0.97	124.56 ± 0.74	_
21 Days	189.73 ± 0.27	263.36 ± 0.23	< 0.05*

GRAPH 1 : UV-VIS spectra of Punicalagin

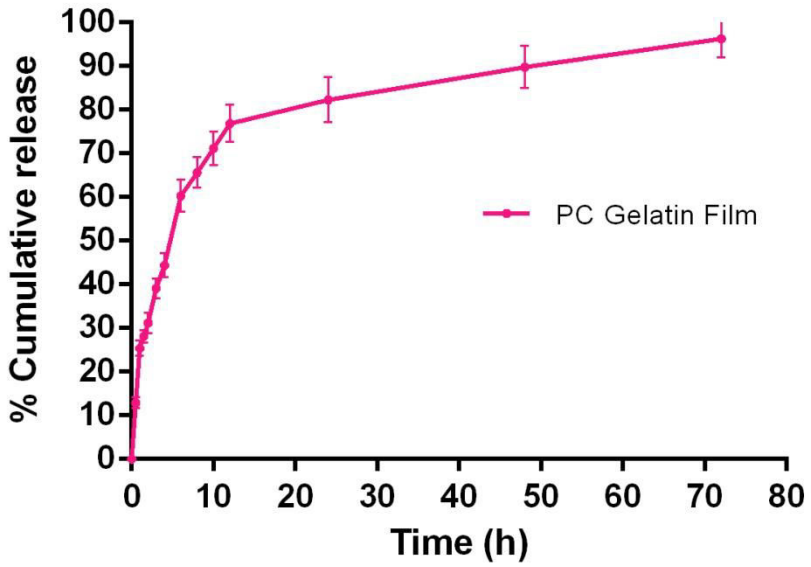


GRAPH 2 : UV-VIS calibration curve of Punicalagin

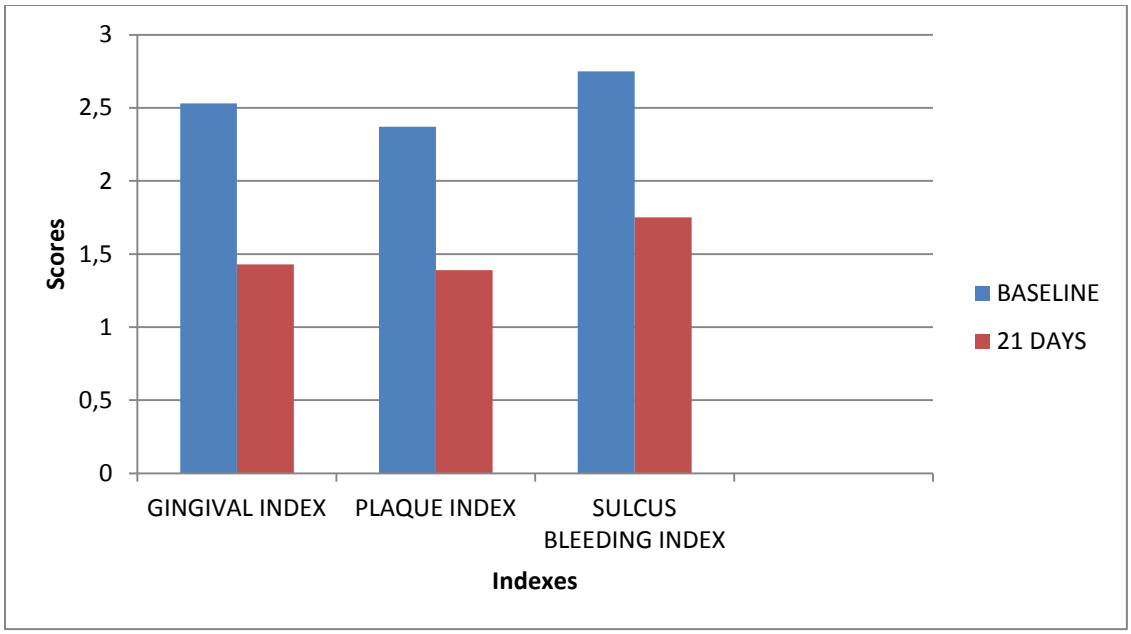


GRAPH 3: Cumulative release profile of PC from Gelatin in buffer pH 6.6.

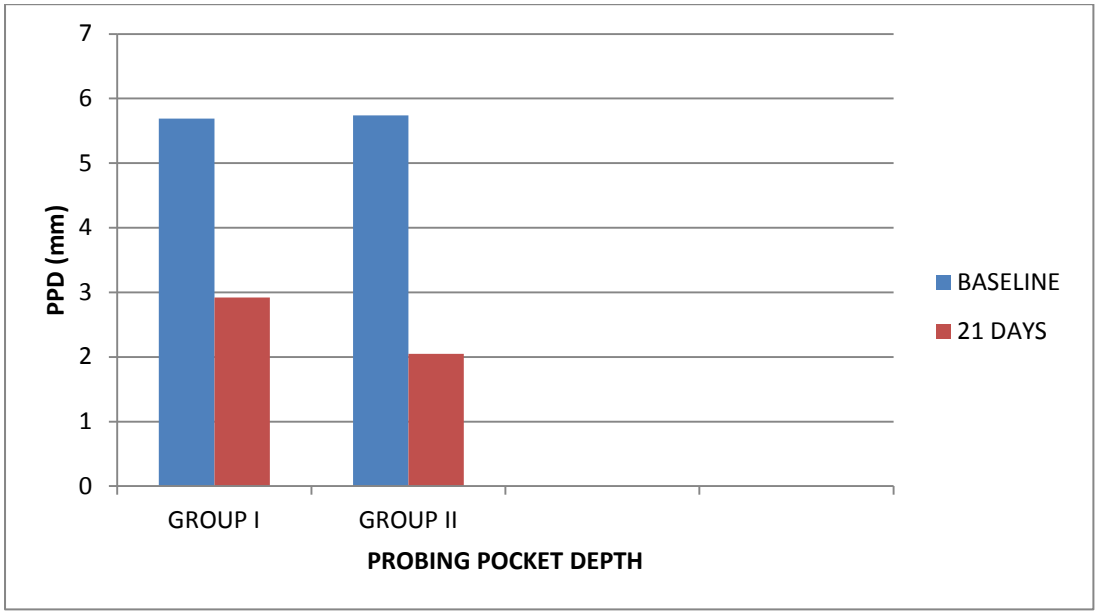
Results represent mean ± SD, n = 3.



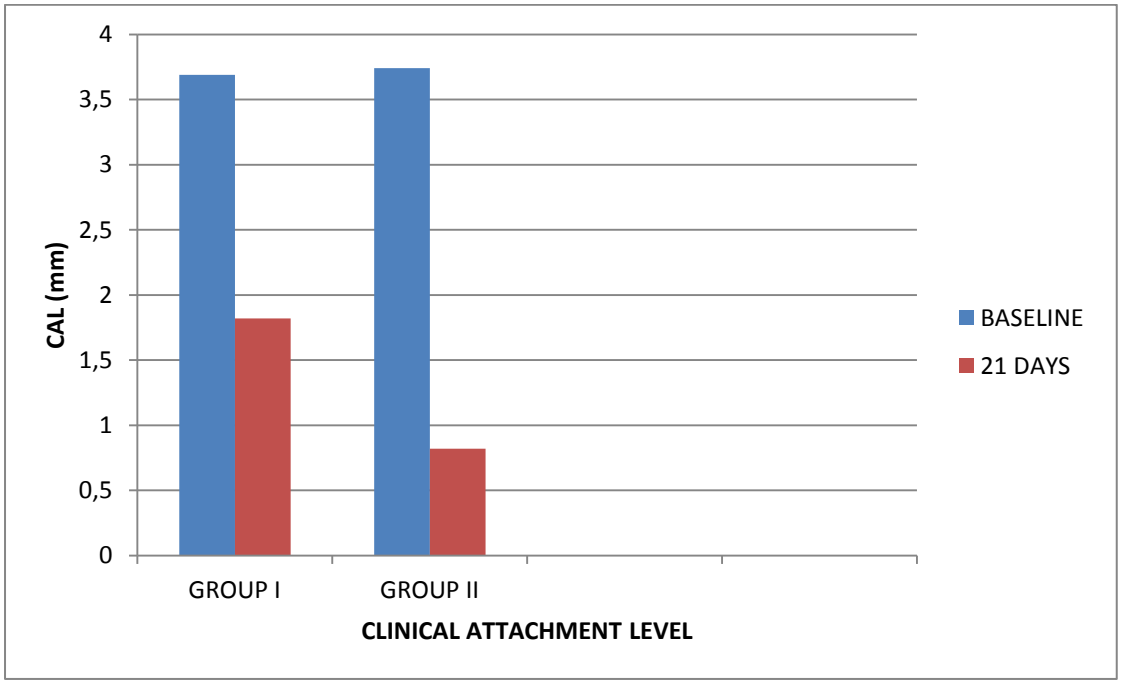
GRAPH 4 : Mean Gingival Index, Plaque Index and Sulcus Bleeding Index at baseline and 21 days post-therapy.



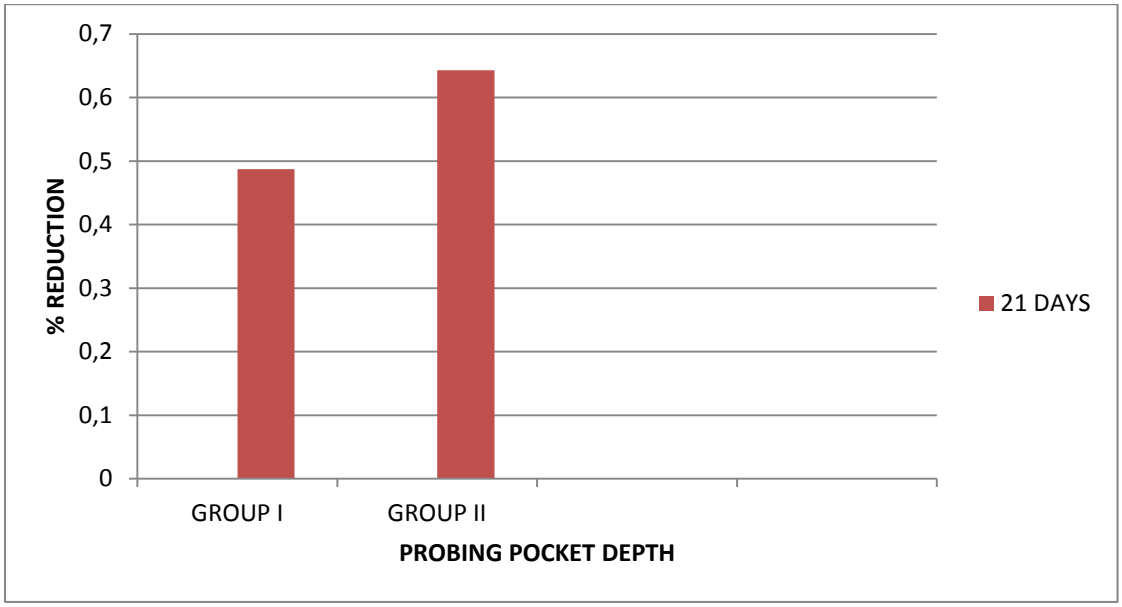
GRAPH 5 : Probing Pocket Depth in both groups at baseline and 21 days post-therapy



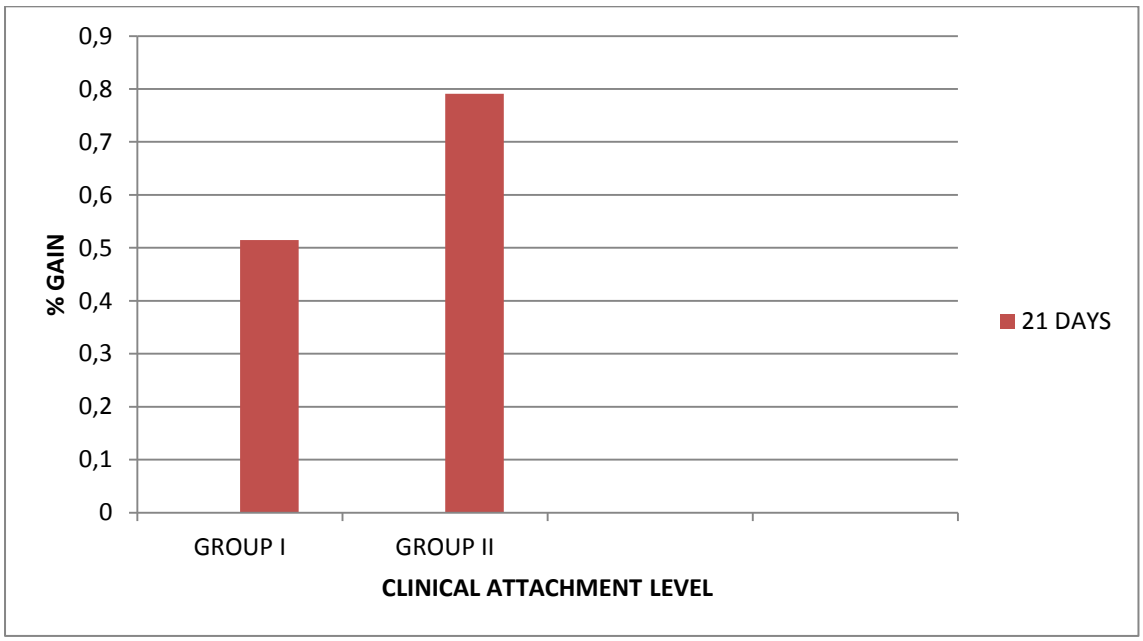
GRAPH 6 : Clinical Attachment Level in both groups at baseline and 21 days post therapy



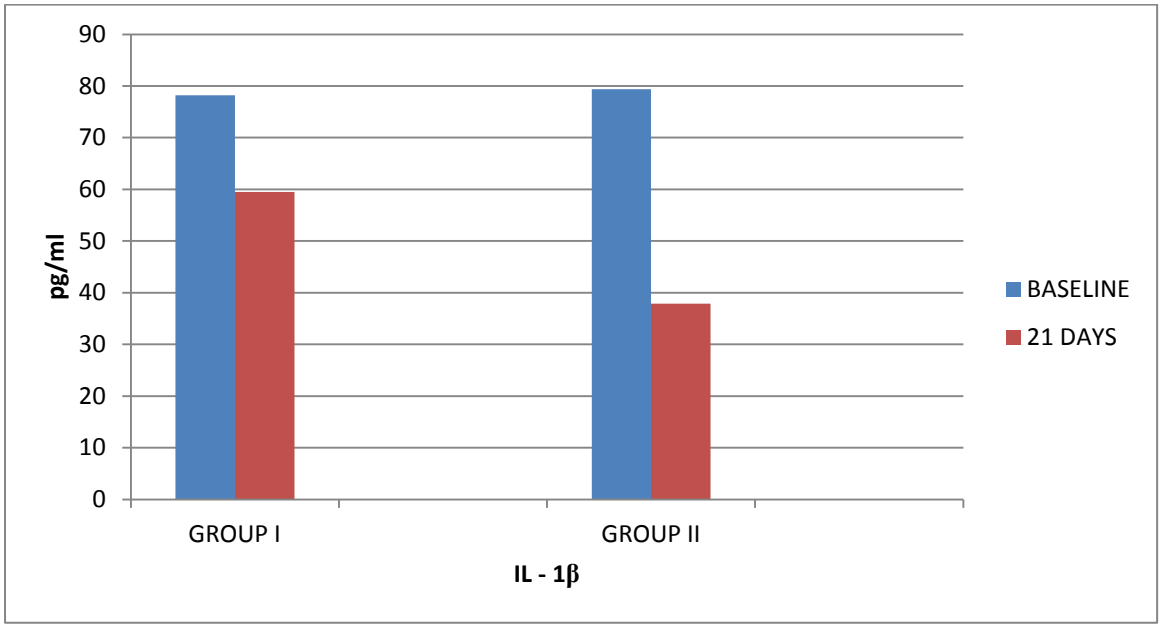
GRAPH 7 : Comparison of % of reduction in Probing Pocket Depth between Group I and Group II post-therapy



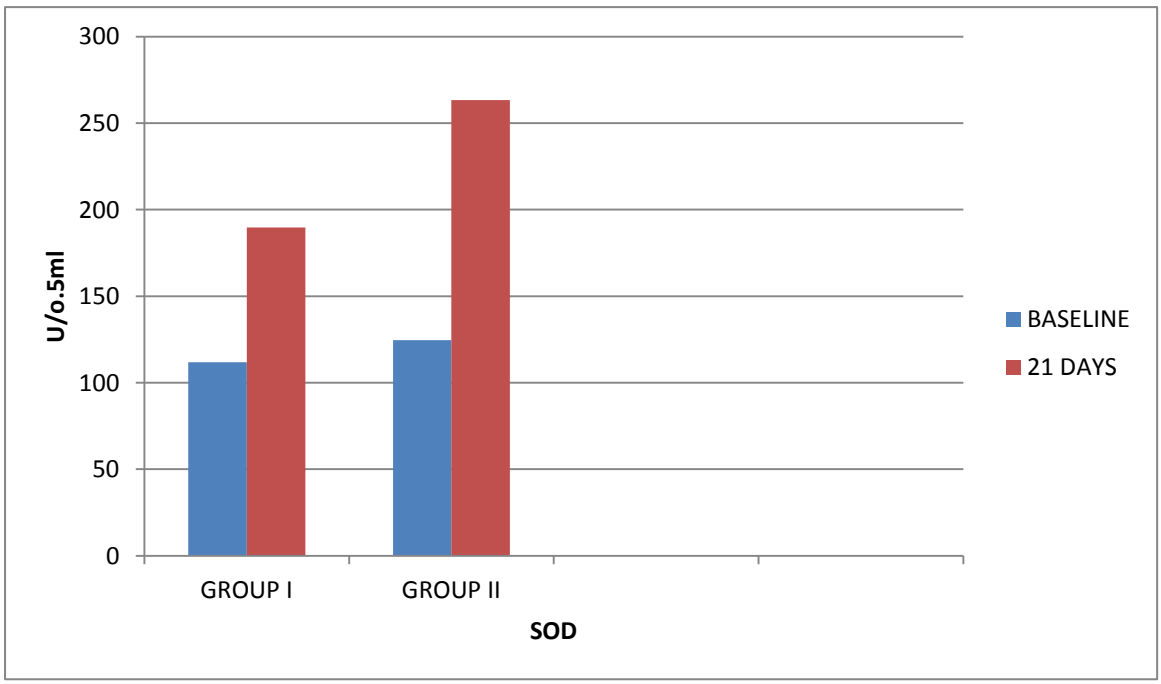
GRAPH 8 : Comparison of % of gain in Clinical Attachment Level between Group I and Group II post-therapy



GRAPH 9 : IL-1β levels in both groups at baseline and 21 days post therapy



GRAPH 10 : Superoxide Dismutase levels in both groups at baseline and 21 days post therapy



The ultimate goal of periodontal therapy is to reconstitute the tissues destroyed by periodontal disease. Scaling and Root Planing has been the ideal choice of procedure to treat periodontal disease but complete elimination of periodontopathogens and tissue destruction is impossible.[56] Local delivery of drugs along with Scaling and Root Planing offers greater advantage of high accessibility at the target site with limited side effects. Punicalagin is the major polyphenol isolated from pomegranate, shown to possess anti-inflammatory and anti-oxidant properties.[46] In the present study, punicalagin is used as a Local Drug Delivery, mode in the form of a gel along with Scaling and Root Planing for the treatment of Chronic Periodontitis patients.

In this study, the mean plaque index score at baseline was found to be 2.37 ± 0.62. After 21 days of treatment, the value was reduced to 1.39 ± 0.58. Statistically significant difference was found from baseline to 21 days post-treatment ($p<0.001$). This was in accordance with the study done by Bhadbhade et al[53] who showed significant reduction in plaque formation following mouth rinsing with Punica granatum mouthwash when compared to placebo. Punicalagin suppresses the ability of the microorganisms to adhere to the surface of the tooth and prevents the formation of plaque. Pomegranate also prevents the ability to adhere by interfering with substances such as extracellular polysaccharides that the bacteria use as a glue.

In the present study, the mean gingival index score at baseline was 2.53 ± 0.67 and reduced to 1.43 ± 0.52 after 21 days post-treatment. Sulcus Bleeding Index score at baseline was 2.75 ± 0.28 and was decreased to 1.75 ± 0.29 after 21 days post-therapy. There was a statistically significant reduction in both Sulcus Bleeding Index and gingival index after 21 days post-treatment ($p<0.001$). This was in

accordance with the study done by Sahgal et al[57] who reported that Punica granatum applied in a gel form was efficient in reducing the Gingival Index score because of its strong styptic action. The reduction in Sulcus Bleeding Index was in accordance with the study by Ahuja et al[58] where pomegranate mouthwash used two times daily for fifteen days resulted in more efficient decrease in bleeding on probing scores when compared to Chlorhexidine mouthwash. He also reported that Pomegranate was effective in reducing both gingival and bleeding on probing scores because of its strong styptic action.

In the present study, group II showed more statistically significant reduction in Probing pocket depth and gain in Clinical Attachment level at 21 days post-therapy when compared to group I. Group I showed Probing Pocket Depth of 5.69 ± 0.00 at baseline which was reduced to 2.92 ± 0.17 at 21 days post-therapy. Group II showed Probing Pocket Depth of 5.74 ± 0.00 at baseline which was decreased to 2.05 ± 0.62 at 21 days post-therapy. Clinical Attachment Loss for group I and group II at baseline were 3.69 ± 0.22 and 3.74 ± 0.38. Clinical Attachment Loss value was reduced to 1.82 ± 0.48 and 0.82 ± 0.22 at 21 days post-therapy respectively. Similar results were shown by Sastravaha et al[50] and reported that pomegranate extract provided significantly better results in decreasing the probing depth and attachment loss. Punicalagin has been shown to provide a synergistic action in collagen stabilization as they have the affinity for proteins and forming bonds with collagen fibers.

In this study, IL-1β levels at baseline were 78.23 ± 0.36 and 79.36 ± 0.18 in group I and group II respectively. Levels were reduced to 59.53 ± 0.22 and 37.9 ± 0.83 in group I and group II respectively at 21 days post treatment. There was

statistically significant decrease in the level of IL-1β after 21 days post-therapy in both the groups ($p<0.001$). When comparing between the groups, Group II showed more statistically significant ($P<0.05$) decrease in the level of IL-1β than group I at 21 days post-therapy. Similar results were observed in a study done by Xu et al[59] who reported that punicalagin reduced the levels of secreted proinflammatory cytokines such as IL-1β, IL-6 and TNF-α. Pomegranate extract exhibited anti inflammatory activity through inhibition of NF-kB activity and prevention of ERK-1 and ERK-2 activation. Pomegranate extract have been shown to inhibit the IL 1 beta induced destruction of proteoglycan, and also inhibits the expression of MMPs, Nitric Oxide and PGE_2 production. Ahmed et al[60] also showed that Pomegranate extract exhibited anti-inflammatory property by inhibiting the IL-1β–induced expression of MMP-1, -3, and -13 mRNA expression. Recently Xu et al[61] showed that Pomegranate extract produced potential anti-inflammatory effect through modulating the synthesis of several mediators and cytokines that are involved in the inflammatory process such as IL-1β and TNF-α

In the present study, SuperOxide Dismutase levels at baseline were 111.83 ± 0.97 and 124.56 ± 0.74 in group I and group II respectively. SuperOxide Dismutase Levels were increased to 189.73 ± 0.27 and 263.36 ± 0.23 in group I and group II respectively at 21 days post-therapy. When comparing between the groups, Group II showed more statistically significant ($P<0.05$) increase in the level of SuperOxide Dismutase than Group I after 21 days post-therapy. This was supported by a study done by Gil et al[62] who showed that high antioxidant activity of Punica granatum was due to the presence of punicalagin isomers, ellagic acid derivatives and anthocyanins. Pomegranate extract inhibits lipopolysaccharide induced oxidative

stress by reducing Reactive Oxygen Species, Nitric Oxide generation and by increasing Superoxide Dismutase 1 mRNA expression.[46]

Based on the above results obtained in this study, there is an improvement in the clinical parameters with an increase in the anti-oxidant marker level and a decrease in inflammatory marker level when punicalagin gel was used along with Scaling and Root Planing in patients with Chronic Periodontitis.

The present study involved a comparative clinical and biochemical evaluation of punicalagin loaded gel along with Scaling and Root Planing (SRP) and SRP alone in patients with Chronic Periodontitis.

Within the framework of this study, the following conclusions have been elucidated,

1. The adjunctive use of Punicalagin in addition to Scaling and Root Planing(SRP) resulted in better treatment response when compared to SRP alone.
2. Probing Pocket Depth reduction and gain in attachment level were significant in both the groups when compared to baseline. When compared between the groups, Group II showed more Probing Pocket Depth reduction and Clinical Attachment Level gain at 21 days post treatment with a statistically significant p value of < 0.001.
3. A decrease in IL-1β levels and an increase in SOD levels were also seen in both the groups with significant difference at 21 days when compared to baseline. When compared between the groups, Group II showed more statistically significant decrease in IL-1β levels and increase in Superoxide Dismutase levels than Group I.

The results showed that punicalagin has the potential to serve as a therapeutic agent to treat chronic periodontitis patients. Compared to Scaling and Root Planing alone, adjunctive use of punicalagin showed greater probing Pocket Depth reduction, Clinical Attachment Level gain and favourable changes in inflammatory and anti-oxidant markers.

However, limitations of this study includes a small sample size and a short term follow up. In order to evaluate long term benefit of punicalagin as a therapeutic agent, a long term study with a larger sample size is required.

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